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# Prognostic Value of the Intratumoral Lymphocyte to Monocyte Ratio and M0 Macrophage Enrichment in the Melanoma Immune Microenvironment

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**Simple Summary:** Melanoma is a deadly skin cancer that is increasing in prevalence yearly. Although most patients with melanoma experience good outcomes, many still do not. Here, we use publicly available gene sequencing data from a large cohort of patients with melanoma to better understand how the “tumor immune microenvironment” (TIME) might influence disease progression and response to therapies. We found that a subset of patients with tumors containing a low lymphocyte:monocyte ratio and high undifferentiated (M0)-macrophage content in the locoregional tumor site have significantly decreased overall survival compared to other groups. Additionally, this same group demonstrates lower predicted rates of response to immune checkpoint blockade therapy and increased rates of metastasis compared to the other groups in the total cohort. Our study may provide a tool to help risk-stratify patients with melanoma, and may lead to the identification of new therapeutic targets for these patients in the future.

**Abstract:** Background: Cutaneous melanoma (SKCM) is characterized by significant heterogeneity in its molecular, genomic, and immunologic characteristics. Methods: Whole transcriptome RNAseq data from The Cancer Genome Atlas of SKCM (n=328) was utilized. The immune microenvironment was characterized using CIBERSORTx to identify immune cell type composition. Unsupervised hierarchical clustering was performed based on immune cell type content. Samples were separated into those obtained from the primary tumor site and regional skin or soft tissue (locoregional), or distant metastasis and regional lymph node (metastatic). Analysis of overall survival (OS) was performed using Kaplan-Meier estimates and Cox-regression multivariable analyses. Results: Four immune clusters were identified, largely defined by lymphocyte:monocyte (L:M) ratio, monocyte-enrichment, and M0-macrophage-enrichment (L:M<sup>Low</sup>, Monocyte<sup>High</sup>, M0<sup>High</sup>; L:M<sup>Low</sup>, Monocyte<sup>Mid</sup>, M0<sup>Low</sup>; L:M<sup>Mid</sup>, Monocyte<sup>Low</sup>, M0<sup>Low</sup>; L:M<sup>High</sup>,

Monocyte<sup>Low</sup>, M0<sup>Low</sup>). The L:M<sup>Low</sup>, Monocyte<sup>High</sup>, M0<sup>High</sup> cluster demonstrated significantly worse OS than clusters 2-4 in the locoregional group (HR 2.804, 95% CI 1.262–6.234, p=0.0114). Membership in the L:M<sup>Low</sup>, Monocyte<sup>High</sup>, M0<sup>High</sup> cluster was an independently poor prognostic factor for survival (HR 3.03, 95% CI 1.12–8.20, p=0.029). The L:M<sup>Low</sup>, Monocyte<sup>High</sup>, M0<sup>High</sup> cluster correlated with higher rates of metastasis and decreased predicted response to immune checkpoint blockade compared to the other clusters as determined by the Tumor Immune Dysfunction and Exclusion tool (TIDE). Conclusion: Distinct tumor immune clusters with a M0-macrophage-enriched, L:M ratio low phenotype in the locoregional melanoma tumor site independently characterize an aggressive phenotype that may differentially respond to treatment.

**Keywords:** Cutaneous Melanoma, Immunotherapy, Lymphocytes, Monocytes, Macrophages, RNAseq, tumor immune microenvironment

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## 1. Introduction

Melanoma is the most aggressive form of skin cancer known, and its incidence continues to rise [1, 2]. It is estimated that by the end of 2020, there will be 100,350 new cases and 6,850 deaths as a result of melanoma [3]. Several factors have been demonstrated to be predictive of poor prognosis in patients with melanoma, including sex, age, ulceration, mitotic rate, and Breslow tumor thickness [4-6]. Fortunately, most patients who present at an early, locoregional stage are potentially curable. On the other hand, patients who present with advanced melanoma have a poor prognosis, with a 5-year survival rate of 10% [7]. There remains a subset of patients, however, that present with locoregional disease but still demonstrate a poor overall survival due to recurrence and disease progression [4-7]. For this reason, it is of paramount importance to identify novel and effective prognostic markers for patients with malignant melanoma.

It has become well-known in recent years that inflammatory responses play key roles in the pathways of tumor initiation, development, and progression [8, 9]. For this reason, hematologic parameters of the inflammatory response have recently become more well-studied, and have been shown to demonstrate prognostic value in various cancers [10, 11]. These hematologic indices include markers such as the C-reactive protein [11, 12], the platelet-to-lymphocyte ratio [13, 14], and the neutrophil-to-lymphocyte ratio [15-17]. However, given the limited prevalence of publicly available whole transcriptome tumor sequencing data, there is a paucity of data on inflammatory parameters within the primary tumor, allowing for exploration of the tumor immune microenvironment (TIME).

Recently, it has been found that a decreased ratio of peripheral lymphocyte-to-monocyte ratio may be a poor prognostic indicator in various cancers [18-23]. The prognostic value of the lymphocyte-to-monocyte ratio may be explained by the fact that lymphopenia is a surrogate marker of weak immune response, while an elevated monocyte count may stand as a surrogate marker of high tumor burden. Even more interestingly, it has previously been demonstrated that this ratio may predict immunotherapy response. Failing, et al. demonstrated in 2017 that a decreased lymphocyte-to-macrophage ratio (<1.7) in the peripheral blood may in fact predict a poor response to pembrolizumab in metastatic melanoma patients [24]. In this analysis, we leverage data from The

Cancer Genome Atlas (TCGA) to explore the landscape of the TIME in melanoma as well as the prognostic impact of the lymphocyte-to-monocyte ratio in whole-exome sequenced tumor specimens, rather than in the peripheral blood, using bulk tumor sequencing data.

## 2. Methods:

### 2.1 Study Design:

We conducted a retrospective analysis of patients with histological diagnosis of Cutaneous Melanoma (SKCM) in the TCGA database. Samples came from institutions across Australia, Brazil, Canada, Georgia, Germany, Moldova, Romania, Russia, the United States, Vietnam, and Yemen. Each patient with clinical and genomic data was independently reviewed by two authors (N.K.J and M.F.). Since all data utilized are available to the public, approval from the Institutional Review Board (IRB) was not required for this analysis. All analyses were performed using the R software for statistical computing (R Version 3.6.3, Vienna, Austria).

### 2.2 Clustering Based on Immune Cell Subpopulations

The CIBERSORT “in silico flow cytometry” tool was used to quantify the relative levels of distinct immune cell types in the TCGA SKCM dataset [25]. A mixture file containing the RNA Seq by Expression Maximization (RSEM) gene expression data from the samples in the TCGA SKCM dataset was downloaded from the cBioPortal and formatted according to the guidelines outlined in the CIBERSORT manual [26, 27]. The LM22 signature genes file was used as a reference point for comparison. LM22 contains 547 genes that accurately distinguish 22 mature human hematopoietic populations and activation states, including seven T cell types, naïve and memory B cells, plasma cells, NK cells and myeloid subsets. The LM22 file was constructed from the gene expression profiles of those cell types measured on Affy U133A/Plus2 and Illumina Expression BeadChip (HumanHT-12 v4) platforms. By default, CIBERSORT estimates the relative fraction of each cell type in the sample, such that the sum of all relative fractions for each of the 22 cell subsets is equal to 1 for the sample.

The package “ComplexHeatmap” was downloaded from Bioconductor [28]. Unsupervised hierarchical clustering was performed by individual patient sample, using lymphocyte and monocyte relative infiltration values, as well as ratios of macrophage subtypes to total macrophages and lymphocyte to monocyte ratio as clustering criteria. Clustering was performed on both x and y-axes to distinguish high vs. low expression values of both clusters and cell types. The clustering distance metric was set to maximum distance between rows, and the clustering method was the Ward’s minimum variance. The dataset was divided into four clusters based on the immune cell subpopulation distribution. Sample IDs corresponding to each cluster were extracted for further analysis.

### 2.3 Survival Analysis of Clusters

The primary outcome in our analysis was overall survival (OS), defined as the time from pathologic diagnosis to death or loss to follow-up, as defined by the TCGA study group. Details including BRAF, NF1, RAS mutations, tumor mutational burden (TMB), % necrosis in the pathologic sample, % tumor content, Breslow depth, occurrence of metastatic disease, age at diagnosis, lymphocyte

score (a surrogate for distribution and density of lymphocytic infiltrate in the tumor sample), timing and dose of therapies, including chemotherapies, immunotherapies, and radiation was obtained from the 'TCGAbiolinks' web tool (R Foundation for Statistical Computing Version 3.6.2, Vienna, Austria). Patients still alive were censored at the time of last follow up. Patients were separated into two groups for Kaplan-Meier analysis: those in whom the sample was taken from a regional lymph node or distant metastasis site (metastasis), compared with those for whom the tumor was taken from the primary site or regional skin or soft tissue (locoregional). Each endpoint was assessed using the Kaplan-Meier method, and survival curves were compared using the Mantel-Cox log-rank test. Survival analysis was carried out using the survminer R package (R Foundation for Statistical Computing Version 3.6.2, Vienna, Austria). Log-rank  $p$ -value and risk tables are displayed on each chart.

#### *2.4 Retrieving Raw Count Data*

The "TCGAbiolinks" package was downloaded from Bioconductor [29-31]. The GDC query function was used to retrieve Illumina HiSeq RNA data from locoregional tumors in the TCGA SKCM dataset and the data was downloaded using the GDCdownload function. Raw count data was normalized, and low count genes were filtered according to the default 25% quantile across all samples. The table of normalized and filtered raw count data was extracted for use in downstream analysis.

#### *2.5 Statistical Analysis*

Clinical, pathologic and molecular characteristics were compared between clusters using statistical methods in R using a chi-square test. The Kaplan Meier method was used to compare endpoints across clusters with the log-rank test. To determine the influence of immunologic and clinicopathologic covariates on OS, a multivariable Cox-regression analysis was performed. Statistical significance was set at  $p < 0.05$ .

#### *2.6 Prediction of Immunotherapy Response – TIDE*

Understanding the relevance of the immune composition of tumors to predicting response to immune checkpoint blockade (ICB) is essential. However, most publicly available transcriptome data does not have corresponding ICB treatment data. To overcome this limitation, the Tumor Immune Dysfunction and Exclusion (TIDE) tool was utilized. TIDE is a web-based tool that uses a gene expression signature to predict response to ICB [32]. Z-score transformed whole transcriptome (RSEM) data was downloaded from the cBio Portal and input into the tool. TIDE performance has been validated on melanoma and NSCLC datasets, making it an ideal choice for our study. Output included response prediction, with lower TIDE score corresponding to better immunotherapy response, and other gene signatures associated with immune dysfunction, including interferon gamma response (IFNG), microsatellite instability (MSI), CD274 (T regulatory cell marker), and T cell dysfunction.

### **3. Results:**

#### *3.1 TIME Clustering and Baseline Demographics:*

All patients with SKCM and available clinical and sequencing data in the SKCM TCGA database (N = 328) were clustered based on relative intratumoral lymphocyte, monocyte, M0-macrophage, M1-macrophage, and M2-macrophage concentrations in each individual sample. The patients were divided into four clusters: cluster 1 (N = 85, 26%, lymphocyte:macrophage [L:M]<sup>Low</sup>, Monocyte<sup>High</sup>, M0<sup>High</sup>), cluster 2 (N = 116, 35%, L:M<sup>Low</sup>, Monocyte<sup>Mid</sup>, M0<sup>Low</sup>), cluster 3 (N = 101, 31%, L:M<sup>Mid</sup>, Monocyte<sup>Low</sup>, M0<sup>Low</sup>), cluster 4 (N = 26, 8%, L:M<sup>High</sup>, Monocyte<sup>Low</sup>, M0<sup>Low</sup>). Full unsupervised hierarchical clustering for 22 immune cell subsets can be visualized in **Figure 1A**. The absolute immune cell infiltration in each cluster was determined by the CIBERSORT absolute immune score output and displayed in **Figure 1B**. Pairwise t-tests were conducted to confirm significant differences between cell types between clusters. All clusters demonstrated significantly different levels of each type of immune cell infiltrate ( $p < 0.05$ ). There were no significant differences between median age, gender, site of primary tumor, specimen site, or median follow up between clusters. Full demographic information can be found in **Table 1**.

### 3.2 TIME Cluster Prognostic Effect

The median follow-up time for all patients was 40 months, and was not significantly different across clusters (35, 34, 38, and 38 months for clusters 1-4 respectively). The primary endpoint of the study, OS, was analyzed for each cluster using Kaplan-Meier estimates and compared using the log-rank test. OS was 21.2 months in Cluster 1, 35.2 months in Cluster 2, 38.1 months in Cluster 3, and 55.5 months in Cluster 4. Patients were separated into locoregional (sample curated from primary tumor or regional skin or soft tissue) or metastatic (distant metastasis or regional lymph node) collection site to understand whether the TIME of the locoregional site might guide tumor behavior. There were no significant differences between clusters in the metastatic site cohort (**Figure 2A**, Global  $p$ -value=0.85). The cluster with the poorest performance in the locoregional site SKCM cohort was cluster 1, as demonstrated by pairwise comparisons, which expressed a L:M<sup>Low</sup>, Monocyte<sup>High</sup>, M0<sup>High</sup> phenotype (**Figure 2B**, Global  $p$ -value=0.03).

When stratifying patients by tumor specimen curated from a locoregional site vs. metastatic site, and grouping clusters to isolate the poorly-performing cluster 1, the metastatic group demonstrated no significant difference between the L:M low, M0-enriched group (cluster 1) and the other clusters in the analysis (**Figure 3A**, HR 0.9919, 95% CI 0.6145-1.601,  $p = 0.974$ ). However, when examining only the locoregional samples, the L:M low, M0-enriched group (cluster 1) demonstrated significantly poorer survival compared to the other groups (**Figure 3B**, HR 2.804, 95% CI 1.262 – 6.234,  $p = 0.0114$ ). These results indicate a poorer OS in patients who have locoregional tumors with M0-macrophage-enriched and L:M low phenotype.

### 3.3 Multivariable Survival Analysis

After establishing that M0-macrophage-enriched, L:M low SKCM in the locoregional tumor site offers the poorest prognosis of the immune subpopulations, a multivariable analysis was performed to determine the independent prognostic impact of TIME clusters on OS in the entire cohort (**Figure 4**). Only patients with complete clinical data were included in the multivariable analysis. Cluster 1 (cluster 4 was reference, HR 3.03, 95% CI 1.12 – 8.20,  $p=0.029$ ) was independently prognostic for the endpoint of OS. Stage II disease (HR 11.89, 95% CI 2.70 – 52.30,  $p=0.001$ ), Stage III disease (HR 31.41,

95% CI 7.35 – 134.20,  $p < 0.001$ ), and Stage IV disease (HR 72.05, 95% CI 11.13 – 466.40,  $p < 0.001$ ) at diagnosis also correlated with significantly worse prognosis in a predictable pattern when compared to the reference Stage I, as did Breslow depth (HR 1.32 per mm increase, 95% CI 1.07 – 1.60,  $p = 0.009$ ). BRAF V600K mutation status also trended toward poorer prognosis (HR 2.47, 95% CI 0.86 – 7.10,  $p = 0.093$ ) but did not reach statistical significance.

### 3.4 TIDE ICB response, Clinical, and Molecular Characteristics

Finally, using the TIDE tool described in methods as well as clinical and molecular data collected by the TCGA study group, we sought to characterize the poorly performing M0-macrophage-enriched, L:M low cluster 1 by predicted response to ICB as well as known poor prognosticators and clinical outcomes. Cluster one demonstrated increased rates of metastasis (51% vs. 40%, 41%, 42% for clusters 2-4 respectively), decreased predicted response to immunotherapies (19% vs. 34%, 35%, 38% in clusters 2-4 respectively), increased rate of BRAF mutations (64% vs. 51%, 44%, 44% in clusters 2-4 respectively), increased % necrosis in the pathologic tumor sample (5.8% vs. 2.7%, 3.2%, 0.8% in clusters 2-4 respectively), increased median Breslow Depth (4.5mm vs. 3mm, 3.2mm, 2.5mm in clusters 2-4 respectively), and decreased lymphocyte score (a surrogate for distribution and density of lymphocytic infiltrate in the tumor sample, 2.2 vs. 2.8, 3, 3.9 for clusters 2-4 respectively). Notably, tumor mutational burden and tumor content were similar across clusters, and cluster 1 demonstrated few RAS mutations and a decreased microsatellite instability score when compared to the other clusters. Full visualization of this data can be seen in **Table 2**.

## 4. Discussion:

With the prevalence of melanoma continuing to rise and the persistence of a subgroup of patients that suffers poor prognosis despite appropriate therapies, it is of increasing importance that novel predictors of therapeutic response and therapeutic targets are developed. In this analysis, we explore the landscape of the TIME in melanoma, and identify unique clusters based on the prevalence of lymphocytes and monocytes within the tumor samples. We demonstrate that a low lymphocyte-to-monocyte (L:M) ratio in the locoregional tumor specimen with high M0-macrophage enrichment confers worse prognosis in SKCM. Additionally, this group displays fewer predicted responders to immunotherapy, concordant with results presented from analyses involving the peripheral blood ratios [24]; poorly prognostic clinical characteristics (higher tumor mutational burden and necrosis, lower lymphocyte score, higher percentage of BRAF mutants), and higher rates of metastatic disease and recurrence. Together, these results suggest that a large undifferentiated macrophage pool in the setting of low lymphocytic infiltrate in the locoregional tumor site may serve to create a favorable environment for tumor progression. It is also possible that undifferentiated macrophages exert an immunosuppressive effect on the tumor, leading to poor lymphocytic infiltration, and thus poorer OS.

Peripheral blood L:M ratio was first found to be a prognostic factor in hematological malignancies [33,34]. Subsequently, a higher L:M ratio has been shown to be associated with improved OS in over a dozen different solid tumors [35]. Most of these studies have been published since 2014, highlighting the recent interest in the prognostic value of L:M ratio in this population. L:M ratio

derived from primary or metastatic tumor sequencing has yet to be investigated, prior to this analysis. The actual mechanisms of the relationship of low L:M ratio and poor outcome of cancer patients are unclear. This association may be explained through tumor-infiltrating immune cells which play a critical role in suppression or enhancement of tumor growth. Tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs) are particularly important immune cells found in tumor and have been found to be important prognostic factors in various cancers [36]. TILs are thought to be responsible for cellular as well as humoral antitumor immune responses that contribute to tumor control. Indeed, high numbers of TILs are associated with improved clinical outcomes [37-39]. In addition, lymphopenia was previously found correlated to overall survival in prospectively collected series of patients with metastatic breast cancer, non-Hodgkin lymphoma, and soft tissue sarcoma [40]. A low lymphocyte count might result in an inadequate immune response in the control of tumor, which may help to explain why a low L:M ratio correlated with poorer OS and increased rates of metastasis, necrosis, and Breslow depth at diagnosis.

TAMs are regarded as key contributors to the crosstalk between tumor and stromal cells, orchestrating key events necessary for cancer progression including skewing adaptive responses, cell growth, angiogenesis, and extracellular matrix remodeling – changes which all lead to a pre-metastatic niche [41, 42]. Further sub-classification of TAMs is necessary as their polarization influences their behavior. At a basic level, macrophages are separated into the M1 subtype which is pro-inflammatory, anti-fibrotic and activated by LPS, TNF, and IFN- $\gamma$  and the M2 subtype which is anti-inflammatory, pro-fibrotic, and stimulated by IL4 and IL13 [43, 44]. Given the dynamic nature of the tumor microenvironment and the numerous stimuli within it [43], emerging classification paradigms describe TAMs on a continuum of many subtypes or a mixed phenotype that is consistent with neither M1 nor M2 phenotypes [45]. Regardless of the phenotype, all TAMs participate in some degree of immunosuppression [46].

In ovarian cancer and glioblastoma, transcriptomic profiling demonstrated that M0 macrophages do not fit into the canonical M1 or M2 model, but M0 macrophages did have high expression of M2 markers, and a transcriptional profile more similar to M2 macrophages [47, 48]. Ultimately, M0s may represent another type of TAM or an incompletely differentiated M2 [47]. M0 macrophages were found to be one of the cell subsets most strongly associated with poor outcome in breast cancer [49], prostate cancer [50], and lung adenocarcinoma [51], while reduced M0 content has been associated with better prognosis in bladder cancer [52]. In a comprehensive analysis of digestive system cancers, M0 macrophages were among the most prevalent immune cell fractions, with M0 enriched clusters associated with decreased recurrence-free survival (RFS) and worse prognostic immune score [53]. It is possible that the presence of these tumor-promoting cells offered more prognostic significance in the locoregional tumor sample in our study due to their promotion of immunosuppression leading to aggressive phenotype and a pre-metastatic niche, which translated to poorer survival. In patients who have already metastasized, prognosis will likely already be poorer, and the relative intratumoral immune cell concentrations at these sites thereby offers little in the way of stratifying prognosis.

With the advent of pembrolizumab, nivolumab, and ipilimumab, the outlook of treatment of advanced melanoma has improved significantly. Patients treated with pembrolizumab in the KEYNOTE-001 study were found to have a median PFS of 4 months and a median OS of 23 months [54]. However, these medications fail to induce a response in a subset of patients, they can cause immune-related adverse events, and they are quite expensive. Therefore, finding biomarkers to help predict which patients are more likely to benefit from treatment is an important area of investigation. Our study found that the cluster of patients with a decreased L:M ratio and M0-macrophage enrichment demonstrated worse OS in the locoregional tumor and in a multivariable analysis accounting for other poor clinical prognostic factors, such as stage, Breslow depth, and BRAF status. Monocytes are recruited into tumors and promote tumor progression [55]. Interleukin-10 is an immunosuppressive cytokine produced mainly by monocytes. In metastatic melanoma, high interleukin-10 levels were correlated with worse survival [56]. Treatment with ipilimumab, nivolumab, or an ipilimumab/nivolumab combination in patients caused significant changes in gene expression in CD3+ T cells, but relatively fewer changes in monocytes [57]. These properties of monocytes may explain our study's finding of why patients with higher baseline M0 macrophage content (and therefore lower L:M ratio) demonstrated poor prognosis and fewer predicted responders to immunotherapy.

Our analysis is a snapshot in time – reflecting when the tumor was resected and sequenced. As a result, the dynamic influences on macrophage polarization and the changing tumor microenvironment were not captured. Additionally, bulk tumor specimen analysis does not capture the immune contexture that is critical to macrophage behavior. Single cell approaches may address some of these issues, however they have the same temporal limitation, are subject to a bias towards more highly expressed genes, they require optimally preserved clinical specimens, and their high cost limits profiling of large numbers of patients [58]. For these reasons, in a clinical decision-making context, an exclusively single cell approach is not feasible. Thus, it is important to use single cell approaches to augment bulk tumor profiling from databases such as TCGA by validating findings from larger scale analyses of bulk specimens. Although we acknowledge that the scope of this study is limited to transcriptome data and that orthogonal studies of corresponding protein expression patterns were not feasible, transcriptome data has become increasingly relevant in the era of molecular medicine. Finally, it will be interesting to examine the significance of the L:M<sup>Low</sup>, Monocyte<sup>High</sup>, M0<sup>High</sup> cluster in a prospective manner based upon directly measured patient responses to immunotherapy.

## 5. Conclusion:

In conclusion, our study has built on the previous literature by characterizing immune clusters within SKCM. With this study we characterize the immune microenvironment landscape of melanoma as it relates to intratumoral lymphocyte and macrophage concentrations, and thereby define a subset of patients with melanoma that display poorer overall outcomes, including decreased OS and fewer ICB responders, based on locoregional tumor biopsy findings. This subset of patients may benefit from more aggressive treatment earlier in the disease course. Additionally, we demonstrate a technique to conduct a comprehensive bioinformatical analysis using publicly available whole transcriptome sequencing data to characterize the tumor immune

microenvironment in cutaneous melanoma and how it relates to clinical outcomes. These results shed light on a potential risk stratification tool, and provide an impetus to further explore the TIME of melanoma, especially as it relates to the locoregional tumor site, and how it may influence disease progression and define novel therapeutic targets in the future.

**Supplementary Materials:** The following are available online at [https://osf.io/3xk6u/?view\\_only=8e316039e80842209742245fc31a8eb2](https://osf.io/3xk6u/?view_only=8e316039e80842209742245fc31a8eb2), supplemental data files and R code repository.

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**References:**

1. Davey RJ, van der Westhuizen A, Bowden NA. Metastatic melanoma treatment: Combining old and new therapies. *Crit Rev Oncol Hematol*. Feb 2016;98:242-53. doi:10.1016/j.critrevonc.2015.11.011
2. Eggermont AM, Spatz A, Robert C. Cutaneous melanoma. *Lancet*. Mar 2014;383(9919):816-27. doi:10.1016/S0140-6736(13)60802-8
3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin*. Jan 2020;70(1):7-30. doi:10.3322/caac.21590
4. Balch CM, Gershenwald JE, Soong SJ, et al. Multivariate analysis of prognostic factors among 2,313 patients with stage III melanoma: comparison of nodal micrometastases versus macrometastases. *J Clin Oncol*. May 2010;28(14):2452-9. doi:10.1200/JCO.2009.27.1627
5. Thompson JF, Soong SJ, Balch CM, et al. Prognostic significance of mitotic rate in locoregional primary cutaneous melanoma: an analysis of patients in the multi-institutional American Joint Committee on Cancer melanoma staging database. *J Clin Oncol*. Jun 2011;29(16):2199-205. doi:10.1200/JCO.2010.31.5812
6. Balch CM, Soong SJ, Gershenwald JE, et al. Age as a prognostic factor in patients with locoregional melanoma and regional metastases. *Ann Surg Oncol*. Nov 2013;20(12):3961-8. doi:10.1245/s10434-013-3100-9
7. Garbe C, Eigentler TK, Keilholz U, Hauschild A, Kirkwood JM. Systematic review of medical treatment in melanoma: current status and future prospects. *Oncologist*. 2011;16(1):5-24. doi:10.1634/theoncologist.2010-0190
8. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002 Dec 19-26 2002;420(6917):860-7. doi:10.1038/nature01322
9. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature*. Jul 2008;454(7203):436-44. doi:10.1038/nature07205
10. Templeton AJ, McNamara MG, Šeruga B, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. *J Natl Cancer Inst*. Jun 2014;106(6):dju124. doi:10.1093/jnci/dju124
11. Shrotriya S, Walsh D, Bennani-Baiti N, Thomas S, Lorton C. C-Reactive Protein Is an Important Biomarker for Prognosis Tumor Recurrence and Treatment Response in Adult Solid Tumors: A Systematic Review. *PLoS One*. 2015;10(12):e0143080. doi:10.1371/journal.pone.0143080
12. Leuzzi G, Galeone C, Gisabella M, et al. Baseline C-reactive protein level predicts survival of early-stage lung cancer: evidence from a systematic review and meta-analysis. *Tumori*. Oct 2016;102(5):441-449. doi:10.5301/tj.5000522
13. Gu X, Gao XS, Cui M, et al. Clinicopathological and prognostic significance of platelet to lymphocyte ratio in patients with gastric cancer. *Oncotarget*. Aug 2016;7(31):49878-49887. doi:10.18632/oncotarget.10490
14. Gu X, Sun S, Gao XS, et al. Prognostic value of platelet to lymphocyte ratio in non-small cell lung cancer: evidence from 3,430 patients. *Sci Rep*. Mar 2016;6:23893. doi:10.1038/srep23893
15. Marchioni M, Cindolo L, Autorino R, et al. High Neutrophil-to-lymphocyte Ratio as Prognostic Factor in Patients Affected by Upper Tract Urothelial Cancer: A Systematic Review and Meta-analysis. *Clin Genitourin Cancer*. 06 2017;15(3):343-349.e1. doi:10.1016/j.clgc.2016.12.027

16. Yin J, Qin Y, Luo YK, Feng M, Lang JY. Prognostic value of neutrophil-to-lymphocyte ratio for nasopharyngeal carcinoma: A meta-analysis. *Medicine (Baltimore)*. Jul 2017;96(29):e7577. doi:10.1097/MD.00000000000007577
17. Huang Y, Sun Y, Peng P, Zhu S, Sun W, Zhang P. Prognostic and clinicopathologic significance of neutrophil-to-lymphocyte ratio in esophageal squamous cell carcinoma: evidence from a meta-analysis. *Onco Targets Ther*. 2017;10:1165-1172. doi:10.2147/OTT.S126637
18. Stotz M, Szkandera J, Stojakovic T, et al. The lymphocyte to monocyte ratio in peripheral blood represents a novel prognostic marker in patients with pancreatic cancer. *Clin Chem Lab Med*. Feb 2015;53(3):499-506. doi:10.1515/cclm-2014-0447
19. Neofytou K, Smyth EC, Giakoustidis A, et al. The Preoperative Lymphocyte-to-Monocyte Ratio is Prognostic of Clinical Outcomes for Patients with Liver-Only Colorectal Metastases in the Neoadjuvant Setting. *Ann Surg Oncol*. Dec 2015;22(13):4353-62. doi:10.1245/s10434-015-4481-8
20. Lucca I, de Martino M, Hofbauer SL, Zamani N, Shariat SF, Klatt T. Comparison of the prognostic value of pretreatment measurements of systemic inflammatory response in patients undergoing curative resection of clear cell renal cell carcinoma. *World J Urol*. Dec 2015;33(12):2045-52. doi:10.1007/s00345-015-1559-7
21. Hutterer GC, Sobolev N, Ehrlich GC, et al. Pretreatment lymphocyte-monocyte ratio as a potential prognostic factor in a cohort of patients with upper tract urothelial carcinoma. *J Clin Pathol*. May 2015;68(5):351-5. doi:10.1136/jclinpath-2014-202658
22. Szkandera J, Gerger A, Liegl-Atzwanger B, et al. The lymphocyte/monocyte ratio predicts poor clinical outcome and improves the predictive accuracy in patients with soft tissue sarcomas. *Int J Cancer*. Jul 2014;135(2):362-70. doi:10.1002/ijc.28677
23. Stotz M, Pichler M, Absenger G, et al. The preoperative lymphocyte to monocyte ratio predicts clinical outcome in patients with stage III colon cancer. *Br J Cancer*. Jan 2014;110(2):435-40. doi:10.1038/bjc.2013.785
24. Failing JJ, Yan Y, Porrata LF, Markovic SN. Lymphocyte-to-monocyte ratio is associated with survival in pembrolizumab-treated metastatic melanoma patients. *Melanoma Res*. 12 2017;27(6):596-600. doi:10.1097/CMR.0000000000000404
25. Newman AM, Steen CB, Liu CL, et al. Determining cell type abundance and expression from bulk tissues with digital cytometry. *Nat Biotechnol*. 07 2019;37(7):773-782. doi:10.1038/s41587-019-0114-2
26. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. May 2012;2(5):401-4. doi:10.1158/2159-8290.CD-12-0095
27. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*. Apr 2013;6(269):pl1. doi:10.1126/scisignal.2004088
28. Gu Z, Eils R, Schlesner M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics*. 09 2016;32(18):2847-9. doi:10.1093/bioinformatics/btw313
29. Colaprico A, Silva TC, Olsen C, et al. TCGAAbiolinks: an R/Bioconductor package for integrative analysis of TCGA data. *Nucleic Acids Res*. 05 2016;44(8):e71. doi:10.1093/nar/gkv1507
30. Silva TC, Colaprico A, Olsen C, et al. : Analyze cancer genomics and epigenomics data using Bioconductor packages. *F1000Res*. 2016;5:1542. doi:10.12688/f1000research.8923.2

31. Mounir M, Lucchetta M, Silva TC, et al. New functionalities in the TCGAblolinks package for the study and integration of cancer data from GDC and GTEx. *PLoS Comput Biol*. 2019;15(3):e1006701. doi:10.1371/journal.pcbi.1006701
32. Jiang P, Gu S, Pan D, et al. Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response. *Nat Med*. 2018;24(10):1550-1558. doi:10.1038/s41591-018-0136-1
33. Li ZM, Huang JJ, Xia Y, et al. Blood lymphocyte-to-monocyte ratio identifies high-risk patients in diffuse large B-cell lymphoma treated with R-CHOP. *PLoS One*. 2012;7(7):e41658. doi:10.1371/journal.pone.0041658
34. Porrata LF, Ristow K, Colgan JP, et al. Peripheral blood lymphocyte/monocyte ratio at diagnosis and survival in classical Hodgkin's lymphoma. *Haematologica*. Feb 2012;97(2):262-9. doi:10.3324/haematol.2011.050138
35. Nishijima TF, Muss HB, Shachar SS, Tamura K, Takamatsu Y. Prognostic value of lymphocyte-to-monocyte ratio in patients with solid tumors: A systematic review and meta-analysis. *Cancer Treat Rev*. Dec 2015;41(10):971-8. doi:10.1016/j.ctrv.2015.10.003
36. Man YG, Stojadinovic A, Mason J, et al. Tumor-infiltrating immune cells promoting tumor invasion and metastasis: existing theories. *J Cancer*. 2013;4(1):84-95. doi:10.7150/jca.5482
37. Mahmoud SM, Paish EC, Powe DG, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol*. May 2011;29(15):1949-55. doi:10.1200/JCO.2010.30.5037
38. Gooden MJ, de Bock GH, Leffers N, Daemen T, Nijman HW. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. *Br J Cancer*. Jun 2011;105(1):93-103. doi:10.1038/bjc.2011.189
39. Fortes C, Mastroeni S, Mannooranparampil TJ, et al. Tumor-infiltrating lymphocytes predict cutaneous melanoma survival. *Melanoma Res*. Aug 2015;25(4):306-11. doi:10.1097/CMR.0000000000000164
40. Ray-Coquard I, Cropet C, Van Glabbeke M, et al. Lymphopenia as a prognostic factor for overall survival in advanced carcinomas, sarcomas, and lymphomas. *Cancer Res*. Jul 2009;69(13):5383-91. doi:10.1158/0008-5472.CAN-08-3845
41. Solinas G, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *J Leukoc Biol*. Nov 2009;86(5):1065-73. doi:10.1189/jlb.0609385
42. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol*. Apr 2010;22(2):231-7. doi:10.1016/j.coi.2010.01.009
43. Xue J, Schmidt SV, Sander J, et al. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity*. Feb 2014;40(2):274-88. doi:10.1016/j.immuni.2014.01.006
44. Capece D, Fischietti M, Verzella D, et al. The inflammatory microenvironment in hepatocellular carcinoma: a pivotal role for tumor-associated macrophages. *Biomed Res Int*. 2013;2013:187204. doi:10.1155/2013/187204
45. Torroella-Kouri M, Silvera R, Rodriguez D, et al. Identification of a subpopulation of macrophages in mammary tumor-bearing mice that are neither M1 nor M2 and are less differentiated. *Cancer Res*. Jun 2009;69(11):4800-9. doi:10.1158/0008-5472.CAN-08-3427

46. Laoui D, Van Overmeire E, Movahedi K, et al. Mononuclear phagocyte heterogeneity in cancer: different subsets and activation states reaching out at the tumor site. *Immunobiology*. Nov 2011;216(11):1192-202. doi:10.1016/j.imbio.2011.06.007
47. Zhang Q, Li H, Mao Y, et al. Apoptotic SKOV3 cells stimulate M0 macrophages to differentiate into M2 macrophages and promote the proliferation and migration of ovarian cancer cells by activating the ERK signaling pathway. *Int J Mol Med*. Jan 2020;45(1):10-22. doi:10.3892/ijmm.2019.4408
48. Gabrusiewicz K, Rodriguez B, Wei J, et al. Glioblastoma-infiltrated innate immune cells resemble M0 macrophage phenotype. *JCI Insight*. 2016 2016;1(2)doi:10.1172/jci.insight.85841
49. Ali HR, Chlon L, Pharoah PD, Markowitz F, Caldas C. Patterns of Immune Infiltration in Breast Cancer and Their Clinical Implications: A Gene-Expression-Based Retrospective Study. *PLoS Med*. Dec 2016;13(12):e1002194. doi:10.1371/journal.pmed.1002194
50. Jairath NK, Farha MW, Srinivasan S, et al. Tumor Immune Microenvironment Clusters in Locoregional Prostate Adenocarcinoma: Prognostic Impact of Macrophage Enriched/Plasma Cell Non-Enriched Subtypes. *J Clin Med*. Jun 2020;9(6)doi:10.3390/jcm9061973
51. Liu X, Wu S, Yang Y, Zhao M, Zhu G, Hou Z. The prognostic landscape of tumor-infiltrating immune cell and immunomodulators in lung cancer. *Biomed Pharmacother*. Nov 2017;95:55-61. doi:10.1016/j.biopha.2017.08.003
52. Li W, Zeng J, Luo B, et al. [High expression of activated CD4. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi*. Feb 2020;36(2):97-103.
53. Yang S, Liu T, Cheng Y, Bai Y, Liang G. Immune cell infiltration as a biomarker for the diagnosis and prognosis of digestive system cancer. *Cancer Sci*. Dec 2019;110(12):3639-3649. doi:10.1111/cas.14216
54. Ribas A, Hamid O, Daud A, et al. Association of Pembrolizumab With Tumor Response and Survival Among Patients With Advanced Melanoma. *JAMA*. Apr 2016;315(15):1600-9. doi:10.1001/jama.2016.4059
55. Chanmee T, Ontong P, Konno K, Itano N. Tumor-associated macrophages as major players in the tumor microenvironment. *Cancers (Basel)*. Aug 2014;6(3):1670-90. doi:10.3390/cancers6031670
56. Torisu-Itakura H, Lee JH, Huynh Y, Ye X, Essner R, Morton DL. Monocyte-derived IL-10 expression predicts prognosis of stage IV melanoma patients. *J Immunother*. 2007 Nov-Dec 2007;30(8):831-8. doi:10.1097/CJI.0b013e318158795b
57. Das R, Verma R, Sznol M, et al. Combination therapy with anti-CTLA-4 and anti-PD-1 leads to distinct immunologic changes in vivo. *J Immunol*. Feb 2015;194(3):950-9. doi:10.4049/jimmunol.1401686
58. Suvà ML, Tirosh I. Single-Cell RNA Sequencing in Cancer: Lessons Learned and Emerging Challenges. *Mol Cell*. 07 2019;75(1):7-12. doi:10.1016/j.molcel.2019.05.003

**Figures and Tables:**

**Figure 1.** Panel A. Heatmap detailing the immune microenvironment landscape of melanoma in The Cancer Genome Atlas. Panel B. Bar plot representing the median absolute immune infiltration separated by cell type and cluster as calculated by CIBERSORT.

**Figure 2.** Kaplan-Meier curves demonstrating the overall survival of all four clusters in The Cancer Genome Atlas cutaneous melanoma cohort. Clusters are color coded, with a legend at the top of the figure. Number at risk at various time points is displayed at the bottom of the figures. Panel A. Kaplan-Meier curve demonstrating survival difference between clusters based on collection from distant metastatic or regional lymph node site. Panel B. Kaplan-Meier curve demonstrating survival difference between clusters based on collection from primary tumor or regional skin or soft tissue site.

**Figure 3.** Panel A. Kaplan-Meier curve displaying overall survival that demonstrates no difference between cluster one (Lymphocyte:Monocyte ratio Low and M0-enriched) and the remaining clusters (grouped) for patients in whom the tissue site sequenced was a distant metastasis or regional lymph node (HR 0.9919, 95% CI 0.6145-1.601,  $p = 0.974$ ). Panel B. Kaplan-Meier curve displaying overall survival that demonstrates a significant difference between cluster one (Lymphocyte:Monocyte ratio Low and M0-enriched) and the remaining clusters (grouped) for patients in whom the tissue site sequenced was a distant metastasis or regional lymph node. Cluster one demonstrated a poorer prognosis (HR 2.804, 95% CI 1.262 – 6.234,  $p = 0.0114$ ).

**Figure 4.** Multivariable cox regression analysis demonstrating the independent prognostic value of important clinical and genetic factors, including gender, age, stage, Breslow depth, BRAF status and cluster designation. Cluster 1 was independently prognostic of overall survival (HR 3.02, 95% CI 1.30 - 7.02,  $p = 0.01$ ).

**Table 1 (Demographics)**

	Cluster 1 (N=85)	Cluster 2 (N = 116)	Cluster 3 (N = 101)	Cluster 4 (N = 26)
Median Age at Diagnosis (Years)	54	57	57	58
Gender				
<i>Male</i>	50	62	57	18
<i>Female</i>	35	54	44	8
Site of Primary Tumor				
<i>Trunk</i>	45	58	59	11
<i>Extremities</i>	31	41	36	12
<i>Head and Neck</i>	7	13	1	3
<i>Other</i>	2	4	3	0
Specimen Site				
<i>Primary Tumor</i>	9	13	14	3
<i>Regional Skin or Soft Tissue</i>	18	20	12	3
<i>Regional Lymph Node</i>	38	39	66	18
<i>Distant Metastasis</i>	13	13	8	2
<i>Not Recorded</i>	5	0	1	0
Median Follow Up (Days)	1060	1010	1136	1133

**Table 2: ICB response, Clinical, and Molecular Characteristics**

	Cluster One (n= 85)	Cluster Two (n=116)	Cluster Three (n=101)	Cluster Four (n=26)
BRAF (%)	64	51	44	44
NF1 (%)	19	16	16	4
RAS (%)	21	34	31	27
TMB	457	482	399	299
Necrosis (%)*	5.8	2.7	3.2	0.8
Tumor Content (%)**	87	84	85	82
Lymphocyte Score***	2.2	2.8	3	3.9
Breslow Depth Median	4.5	3	3.2	2.5
Any Metastatic Disease (%)	51	40	41	42
Metastatic Recurrence (%)	42	34	35	38
TIDE ICB Response (%)	19	34	35	27
TIDE Raw Score	0.54	0.24	-0.6	0.35

IFNG response Score	-0.27	0.06	0.05	0.52
MSI Score	0.41	0.54	0.49	0.51
CD274 Score	0.06	0.03	-0.03	0.05
T Cell Dysfunction	-0.15	-0.17	-0.06	0.62

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\*Continuous variables are reported as median values, except for necrosis, reported as a mean value

\*\*Tumor content represents percentage of malignant cells in the tumor sample

\*\*\*Lymphocyte score is reported as calculated by the TCGA study group

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Table demonstrating the molecular landscape, clinical factors, and markers of immune response by cluster in The Cancer Genome Atlas. Abbreviations: TMB, Tumor Mutational Burden; TIDE, TIDE, Tumor Immune Dysfunction and Exclusion; ICB, Immune Checkpoint Blockade; IFNG, Interferon Gamma; MSI, Microsatellite Instability.